EXHIBIT A

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Tumor targeting by conjugation of DHA to paclitaxel

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Abstract

Targeting an anti-cancer drug to tumors should increase the Area Under the drug concentration–time Curve (AUC) in tumors while decreasing the AUC in normal cells and should therefore increase the therapeutic index of that drug. Anti-tumor drugs typically have half-lives far shorter than the cell cycle transit times of most tumor cells. Tumor targeting, with concomitant long tumor exposure times, will increase the proportion of cells that move into cycle when the drug concentration is high, which should result in more tumor cell killing. In an effort to test that hypothesis, we conjugated a natural fatty acid, docosahexaenoic acid (DHA), through an ester bond to the paclitaxel 2′-oxygen. The resulting paclitaxel fatty acid conjugate (DHA-paclitaxel) does not assemble microtubules and is non-toxic. In the M109 mouse tumor model, DHA-paclitaxel is less toxic than paclitaxel and cures 10/10 tumored animals, whereas paclitaxel cures 0/10. One explanation for the conjugate's greater therapeutic index is that the fatty acid alters the pharmacokinetics of the drug to increase its AUC in tumors and decrease its AUC in normal cells. To test that possibility, we compared the pharmacokinetics of DHA-paclitaxel with paclitaxel in CD2F1 mice bearing ~125 mg sc M109 tumors. The mice were injected at zero time with a bolus of either DHA-paclitaxel or paclitaxel formulated in 10% cremophor/10% ethanol/80% saline. Animals were sacrificed as a function of time out to 14 days. Tumors and plasma were frozen and stored. The concentrations of paclitaxel and DHA-paclitaxel were analyzed by LC/MS/MS. The results show that DHA targets paclitaxel to tumors: tumor AUCs are 61-fold higher for DHA-paclitaxel than for paclitaxel at equitoxic doses and eight-fold higher at equimolar doses. Likewise, at equi-toxic doses, the tumor AUCs of paclitaxel derived from i.v. DHA-paclitaxel are 6.1-fold higher than for paclitaxel derived from i.v. paclitaxel. The tumor concentration of paclitaxel derived from i.v. paclitaxel drops rapidly, so that by 16 h it has fallen to the same concentration (2.8 μM) as after an equi-toxic concentration of DHA-paclitaxel. In plasma, paclitaxel AUC after an MTD dose of DHA-paclitaxel is approximately 0.5% of DHA-paclitaxel AUC. Thus, the increase in tumor AUC and the limited plasma AUC of paclitaxel following DHA-paclitaxel administration are consistent with the increase in therapeutic index of DHA-paclitaxel relative to paclitaxel in the M109 mouse tumor model. A phase I clinical study has been completed at The Johns Hopkins Hospital to evaluate the safety of DHA-paclitaxel in patients with a variety of solid tumors. Twenty-one patients have been treated to date. The recommended phase II dose is 1100 mg/m², which is equivalent to 4.6 times the maximum approved paclitaxel dose on a molar basis. No alopecia or significant peripheral neuropathy, nausea, or vomiting have been observed. Asymptomatic, transient neutropenia has been the primary side effect. Eleven of 22 evaluable phase I patients transitioned from progressive to stable disease, as assessed by follow-up CT. Significant quality of life improvements have been observed. Thus, DHA-paclitaxel is well tolerated in patients and cures tumors in mice by targeting drug to tumors.

Keywords: Tumor targeting; DHA; Paclitaxel

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1. Introduction

Many anti-cancer drugs kill cycling cells in either the S or G2/M phases of the cell cycle while sparing quiescent, mostly normal, cells in G1 or G0 [1]. The fraction of a tumor's cells that are cycling at any time varies depending upon tumor type and the growth stage of the tumor. In general, faster cycling tumors (e.g., lymphomas, testicular tumors, and some childhood tumors) are more susceptible to chemotherapy than are the more common types of solid tumors with slowly or non-cycling cells [1]. Since some normal cells such as bone marrow and intestinal mucosa also cycle rapidly, they often become dose-limiting for therapy [1].

The differences in cell kinetics between slowly and rapidly cycling tumor cells, combined with the upper dose limits imposed on anti-tumor drugs by proliferating normal cells, may partially explain the rather modest success that even new chemotherapeutic drugs have achieved against solid tumors.

One key to more successful chemotherapy is to find some property of tumor biochemistry or physiology that can be exploited to target drugs to tumors and create a greater therapeutic advantage. Tissue-isolated hepatomas with a single arterial inflow and a single venous outflow have been used to study the uptake of fatty acids and other metabolic precursors [2–5]. In these systems, some, but not all, natural fatty acids are avidly taken up by tumors from the arterial blood, presumably for use as biochemical precursors and energy sources [2–5]. We hypothesized that conjugating DHA to paclitaxel might create a drug that targets tumors, producing a higher concentration of cytotoxic drug in the tumors for longer times.

To test this hypothesis, we synthesized DHA-paclitaxel, a 2′-*O*-acyl conjugate of the natural fatty acid docosahexaenoic acid and paclitaxel

Paclitaxel, a complex taxane diterpene, is isolated from various members of the genus *Taxus* [6], and is the active compound in *Taxol*® distributed by Bristol Myers Squibb. *Taxol*® is one of the most effective and widely-used anti-cancer drugs. It has been approved for use in the United States against ovarian, breast, and lung cancers and Kaposi's sarcoma. Paclitaxel shifts the dynamic tubulin–microtubule equilibrium toward the polymeric state by binding to and stabilizing microtubules [7]. Cells treated with paclitaxel develop arrays of parallel bundles of microtubules, and are blocked at the G2/M phase of the cell cycle [8] and die, presumably because the tubulin required to form a functional mitotic spindle is sequestered in the form of stable microtubules.

DHA is an omega-3, C22 natural fatty acid with six *cis* double bonds. It is a constituent of membranes and is used as a precursor for metabolic and biochemical pathways. DHA is found in human milk, is added to infant formula in Europe, and is classified as a nutritional additive by the US Food and Drug Administration. Based on its safety, DHA and its metabolites should contribute little if any additional toxicity to paclitaxel.

2. Methods

DHA-paclitaxel (Fig. 1) was synthesized from paclitaxel and DHA in a single step which covalently links DHA to paclitaxel at the 2′-hydroxyl position. Acylation at the 2′ position has been shown to eliminate the in vitro microtubule assembly activity of paclitaxel [9], and therefore inactivates the cytotoxicity of DHA-paclitaxel until it is metabolized back to paclitaxel or perhaps another cytotoxin. Despite the hydrophobic fatty acid attached to DHA-paclitaxel, the compound is more soluble in 10% *Cremophor* EL-P/10% ethanol/80% saline than is paclitaxel.

3. Results

Range finding studies in our laboratory showed

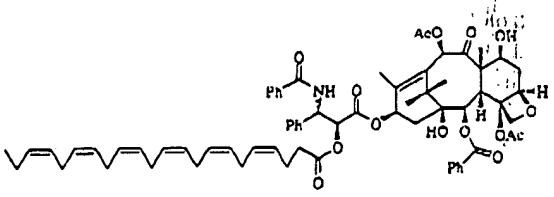

Fig. 1. Structure of DHA-paclitaxel.

that the Optimum Dose (OD). defined as the dose that causes the best therapeutic activity without formulation problems or drug-related deaths, for paclitaxel in female CD2F1 mice is 20 mg/kg in 10% *Cremophor* EL-P/10% ethanol/80% saline delivered through the tail vein once a day for 5 days. The OD for DHA-paclitaxel in female CD2F1 mice is 120 mg/kg in 10% *Cremophor* EL-P/10% ethanol/80% saline delivered through the tail vein once a day for 5 days.

The results show that paclitaxel cured none of the M109 tumors at any dose up to the OD of 20 mg/kg for 5 days (Fig. 2). The growth of the tumor was slowed for about 10 days and then continued at the same rate as the untreated control.

In contrast, DHA-paclitaxel eliminated all measurable tumor masses in 10/10 mice at the OD of five daily doses of 120 mg/kg, in 9/10 mice at 90 mg/kg×5 days, and in 4/10 mice at 60 mg/kg×5 days (Fig. 2). Thus, DHA-paclitaxel eliminates tumors at doses that are 50 and 75% of the OD. These findings predict significant anti-tumor activity in man at less toxic doses.

To test the hypothesis that DHA conjugation to paclitaxel increases anti-tumor efficacy because of drug targeting, we studied the pharmacokinetics of paclitaxel derived from a single i.v. dose of DHA-paclitaxel in tumor-bearing mice. DHA-paclitaxel was injected through the tail vein of mice bearing M109 tumors weighing approximately 100–200 mg. The concentration in the tumors of paclitaxel derived from DHA-paclitaxel rises over 72 h to about 5 μM and then gradually falls to about 1 μM at 14 days. In contrast, paclitaxel derived from i.v. paclitaxel rises and falls rapidly. The tumor AUC of DHA-paclitaxel is 61-fold higher than the tumor AUC of paclitaxel derived from i.v. paclitaxel at equitoxic doses and eight-fold higher at equi-molar doses.

4. Conclusions

One of the intractable problems in cancer therapy is that many tumor cells divide slowly or not at all [1]. Since most anti-cancer drugs only kill dividing cells and have short half-lives in tumors, such drugs can only kill the sub-set of dividing cells in that tumor for the short times the drug concentrations are high enough to be cyto-lethal. DHA-paclitaxel, because of its tumor targeting action, may help to solve those problems as suggested by the M109 efficacy results above. A high concentration of non-cytotoxic DHA-paclitaxel in the M109 tumors that is slowly converted to cyto-lethal concentrations of paclitaxel, or other cytotoxic metabolites, during prolonged times should increase the fraction of both normally and slowly cycling M109 tumor cells killed. Furthermore, once a certain fraction of cells are eliminated, those M109 cells that are the equivalent of non-dividing 'tumor stem cells' [1], may also begin to cycle and be killed. Tumor targeting, then, through conjugation of DHA to paclitaxel, may eliminate those non-cycling cells unresponsive to typical anti-tumor drugs with short half-lives in tumors. The

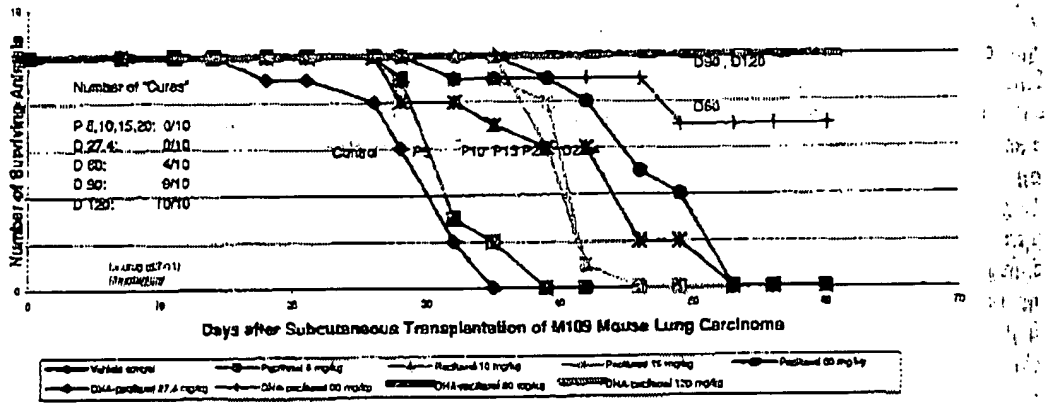

Fig. 2. Number of surviving mice bearing M109 lung carcinomas after treatment with paclitaxel and DHA-paclitaxel.

result is an increase of the apparent cure rate in the M109 model from 0/10 for paclitaxel to 10/10 for DHA-paclitaxel.

A Phase I clinical study at the Johns Hopkins Hospital evaluated the safety profile of DHA-paclitaxel in humans. The Phase II dose will be 1100 mg/m^2.

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